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Review

Lectin chaperones help direct the maturation of glycoproteins in the endoplasmic reticulum

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ABSTRACT

Eukaryotic secretory pathway cargo fold to their native structures within the confines of the endoplasmic reticulum (ER). To ensure a high degree of folding fidelity, a multitude of covalent and noncovalent constraints are imparted upon nascent proteins. These constraints come in the form of topological restrictions or membrane tethers, covalent modifications, and interactions with a series of molecular chaperones. N-linked glycosylation provides inherent benefits to proper folding and creates a platform for interactions with specific chaperones and Cys modifying enzymes. Recent insights into this timeline of protein maturation have revealed mechanisms for protein glycosylation and iterative targeting of incomplete folding intermediates, which provides nurturing interactions with molecular chaperones that assist in the efficient maturation of proteins in the eukaryotic secretory pathway.

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Anfinsen's seminal studies demonstrated that proteins contain all the information in their primary sequence required to fold into their proper three-dimensional structure [1]. Yet elevated temperatures and protein concentrations reaching 300 mg/ml provide unfavorable folding conditions in live cells. Strikingly, protein folding that is reconstituted in the test tube is generally less efficient than in the cell because cells utilize alternative mechanisms, contain factors that assist proteins in reaching their native states and destroy proteins that fail to fold properly. Approximately one third of the entire eukaryotic proteome is predicted to traffic through the secretory pathway [2,3]. For these proteins, protein folding and maturation begin co-translationally as the protein is translocated into the endoplasmic reticulum (ER) lumen. The ER functions as a protein folding factory, harboring a large array of molecular chaperones, oxidoreductases and quality control machinery. The maturation process that includes covalent modifications, processing, folding and assembly continues post-translationally until the native structure is acquired. Cellular protein folding assistance occurs via a number of constraints imparted upon the maturing protein to streamline the folding pathway. This results in a restriction of the vast number of possible conformations and avoidance of off-pathway intermediates to promote the efficient acquisition of the native state. The fidelity of the maturation process is monitored by quality control machinery that sorts native proteins for anterograde trafficking and defective proteins for retention and subsequent destruction.

1. Co-translational translocation and folding in the ER

Protein folding in living cells can begin during synthesis [4,5]. The 'Levinthal paradox' demonstrates that protein folding is not a random walk since there is insufficient time to sample all possible conformations available for a protein to reach its native state [6]. Protein folding in the cell is subjected to a variety of temporal, spatial and physical constraints, providing important restrictions that support a more direct route to the native state. A co-translational folding process creates a vectorial folding reaction and provides a mechanism for the cell to control and organize the environment for the vulnerable nascent chain [7,8]. Tethering the termini of the nascent chain to the ribosome and the membrane further limits the freedom of the nascent chain or the number of available folding intermediates. In addition, the bulky ribosome separates nascent chains of a polysome, preventing nonproductive collisions or aggregation. Together, these mechanisms act to optimize the folding reaction in the apparent suboptimal folding conditions within the cell.

Coupling protein folding and synthesis tapers the possible ensemble of folding intermediates available to the shorter nascent chains during the progression to the native state, thereby minimizing the available nonproductive options [9]. By allowing folding to commence co-translationally or prior to the completion of synthesis, the amount of conformational space that may be sampled by the nascent chain is greatly reduced, allowing short distance or local folding events to occur first. This early restriction in nascent chain folding streamlines the folding pathway by reducing the conformational heterogeneity that is the result of a long fully synthesized unfolded protein precursor [10]. In addition, by initiating folding prior to the completion of synthesis, aggregation and non-native

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interactions of nascent chains are prevented since the constraints also reduce the mobility or diffusion rate of unfolded precursors in the ER [4,9,11]. Thus, the vectorial nature of co-translational folding helps to fold proteins rapidly and efficiently.

Signal sequences on proteins destined for the ER are generally located at the N-terminus of proteins and are first recognized by the cytosolic signal recognition particle (SRP) [12]. SRP delivers the ribosome/mRNA complex to the surface of the ER through interactions with the SRP receptor. Here, the ribosome associates with translocon machinery, facilitating entry of the nascent chain into the ER lumen. Protein translocation into the ER occurs through Sec61, a large multi-protein complex that interacts with the ribosome and allows nascent proteins to transit through its pore [13,14]. The ribosome, Sec61 and their associated proteins can create a privileged environment that sterically protects the nascent chain from nonproductive interactions to favor proper protein maturation [15].

The hydrophobic nature of signal sequences supports their integration into ER membranes. This places a constraint on the maturing protein by tethering its N-terminus to the membrane and restricting the degree to which nascent chains can sample conformational space. This allows newly synthesized proteins to fold in a controlled manner that protects against premature folding events that could result in nonproductive local minima until the signal sequence is cleaved. Signal sequence cleavage is critical for proper protein maturation. In the case of the viral envelope protein, vesicular stomatitis virus glycoprotein G, the failure of the signal sequence to be cleaved results in the ER retention of the misfolded viral protein [16]. The timing and efficiency of signal sequence cleavage is substrate specific; though it generally appears to occur co-translationally, after the synthesis of ~140 amino acids [17–19]. However, the delayed post-translational cleavage of signal sequences has been implicated in the functional activity of a number of proteins including human cytomegalovirus protein US11 and human immunodeficiency virus glycoprotein 120 [20,21]. Furthermore, the timing of co-translational folding events has been proposed to be dictated in part by signals embedded within the amino acids of the signal sequence, likely controlled by directing the timing of signal sequence cleavage and release of the membrane tethering constraint [22].

While local structures such as α -helices have been shown to form in the ribosome and translocon tunnels, global folding occurs upon entrance into the ER lumen through the Sec61 translocon [5,23–26]. Covalent constraints such as disulfide bond formation and modification with sugar chains occur as proteins are being synthesized, and these modifications can also significantly contribute to the efficient maturation of secretory cargo [5,27].

2. N-linked glycosylation

The glycosidic linkage of preformed 14-member carbohydrate chains onto Asn residues in the consensus sequence Asn-X-Ser/Thr (where X can be any amino acid except Pro) are added to the majority of proteins that traverse the secretory pathway [28–30]. Glycans can greatly impact the folding of these proteins. The N-linked glycan is comprised of two *N*-acetyl glucosamines (GlcNAc) and nine mannoses (Man) arranged in three branches (A, B, and C) with three glucoses (Glc) attached to the A-branch mannose residue (Fig. 1). The N-glycan is transferred preassembled from dolichol-P-linked lipids by the oligosaccharyltransferase (OST) that is associated with the Sec61 translocon (Fig. 2, step 1) [28]. There are two forms of the OST that contain different isoforms of the catalytic subunit, STT3 [31]. A recent study has shown that the STT3A-containing OST can support co-translational glycosylation of nascent glycoprotein substrates, whereas the STT3B isoform can additionally facilitate the post-translational glycosylation of substrates [32]. These results suggest that the addition of glycans does not necessarily occur in linear succession.

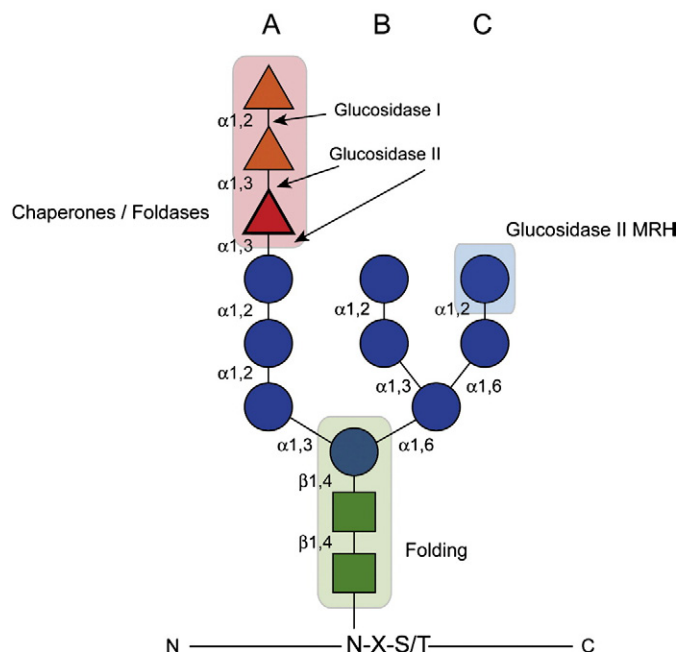


Fig. 1. The composition and roles of N-linked glycans in the ER. The 14-member carbohydrate is covalently linked to Asn residues of the consensus sequence Asn-X-Ser/Thr as depicted. The glycan is comprised of three glucoses (triangles), nine mannoses (circles), and two *N*-acetylglucosamines (squares). The mannoses are arranged in three branches: A, B, and C. The orientations of the glycosidic bonds are indicated. The positions at which glucosidases I and II cleave the glucoses are designated. The glucose that is transferred through GT1 activity and is involved in lectin chaperone binding is highlighted. The regions of the N-glycan that are critical for chaperone interaction, glucosidase II MRH binding, and folding kinetics are shaded.

In addition, the OST subunit Ost3/6p has been found to contain an active thioredoxin-like domain possessing oxidoreductase activity [33]. Since oxidation of substrate proteins has been shown to negatively impact glycosylation efficiency [34,35], the covalent tethering of nascent chains to the Ost3/6p subunit can impact glycosylation transfer efficiency [33].

The addition of N-glycans to nascent proteins has an immediate effect on the folding pathway of the substrate and its eventual fate. N-glycans are highly flexible and hydrophilic structures that mask hydrophobic stretches on proteins and greatly impacting the sampling of conformational space during folding [36,37]. Examples are available that demonstrate the intrinsic effects of glycosylation on protein stability and oligomerization, with glycans providing a chaperone-like effect on protein folding [38]. N-glycans have been shown to prevent the undesired association of hydrophobic stretches, which can lead to aggregation [39,40]. Indeed, glycosylation has been implicated in maintaining the prion protein in a non-fibril conformation [41]. The flexibility of N-glycans is exploited to protect proteins from proteases and shield pathogens from immune detection [42–44]. Recent studies have revealed tight interactions between the base trisaccharide core (Man₁GlcNAc₂) and the folding polypeptide that impacts the kinetics of protein folding [45]. The majority of this contribution to protein folding thermodynamics and kinetics is actually derived from the single innermost GlcNAc residue. Analysis of the of the Human Gene Mutation Database has revealed that gain-of-glycosylation mutations occur in a much higher prevalence than previously anticipated, while mutation of glycosylation consensus sites are rarer than predicted [46]. This finding highlights the positive influence of the inherent properties of glycans on protein folding in the cell. The mutational introduction of a glycosylation site into a protein can effectively destabilize the unfolded state by restricting conformational sampling, coaxing the unfolded protein towards native state conformations [45].

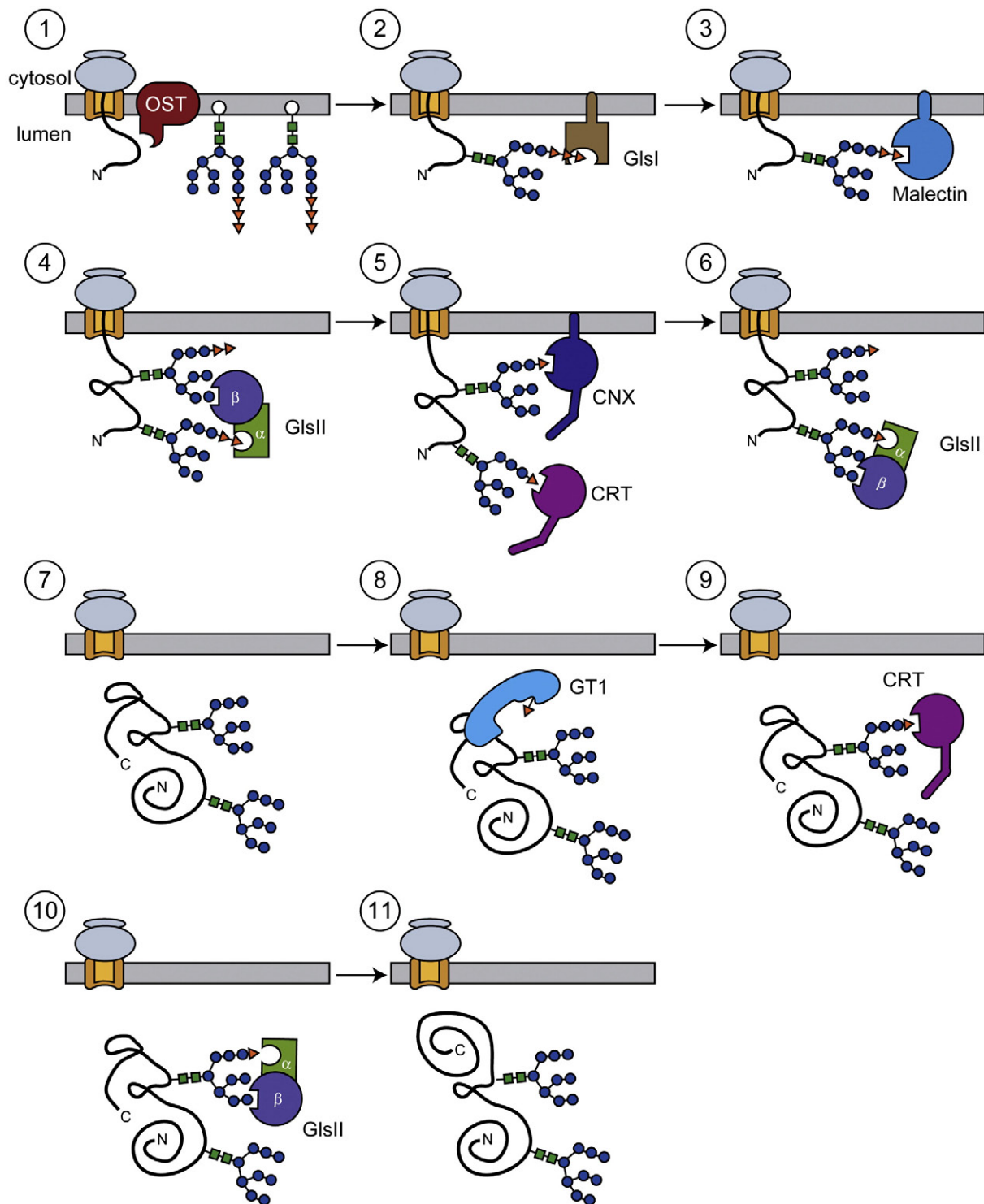


Fig. 2. Modification of N-glycans during glycoprotein maturation. Nascent secretory proteins co-translationally enter the ER lumen. At this time, the membrane integrated oligosaccharyltransferase (OST) transfers 14-member carbohydrates to nascent chains from lipid-linked precursors (step 1). Nearly immediately following glycan transfer, the membrane-associated glucosylase I (Glc I) removes the terminal glucose, generating a $\text{Glc}_2\text{Man}_6\text{GlcNAc}_2$ glycoform (step 2). The exposure of the second glucose allows the type I membrane protein and lectin, malectin, to associate with nascent chains (step 3). In direct competition with malectin binding is the removal of the second glucose by glucosylase II. Glucosylase II is comprised of an MRH domain containing β -subunit and an enzymatic α -subunit. The β -subunit MRH domain docks glucosylase II to glycoproteins via interactions with auxiliary glycans. This in turn allows the α -subunit to remove the second glucose of the target glycan (step 4). Exposure of the innermost glucose permits interactions with the lectin chaperones calnexin and calreticulin (step 5). However, glucosylase II competes with the lectin chaperones for binding to the substrate glycans. In this step, glucosylase II liberates the final glucose via docking of its MRH subunit to mannoses within the target glycan (step 6). The fully deglycosylated glycan is incapable of being bound by the lectin chaperones and is free to fold to its native state (step 7). If non-native conformations persist, the folding sensor GT1 docks to misfolded regions and reglucosylates target glycans (step 8). This results in reassociation with lectin chaperones for additional rounds of folding or retention (step 9). Eventually, the glucose that was added by GT1 is removed by glucosylase II (step 10). At this point, the protein is once again free to fold to its native state. If properly folded, it will traffic to its final functional destination. It may re-enter the lectin chaperone cycle via GT1 activity if non-native conformations remain present. If terminally misfolded, it will eventually be sorted for retrotranslocation and degradation (step 11).

In addition to the inherent influence of N-glycans on protein folding, glycans can also recruit folding factors to assist in protein maturation depending upon the composition of the N-linked glycans [29,47]. After the transfer of the 14-member carbohydrate by the OST, the first glucose is removed by the membrane-bound glucosidase I (Figs. 1 and 2, step 2). This deglycosylation reaction occurs very rapidly, since it can be detected concomitantly with glycan transfer [48]. The Glc₂Man₉GlcNAc₂ glycan composition prevents reassociation with the OST, acting as a commitment step to commence folding [49,50]. Until recently, there were no known ER proteins that interacted exclusively with the diglycosylated glycoform. However, recent studies have identified malectin as a highly conserved ER-localized membrane-bound Glc₂-specific lectin with unknown roles in glycoprotein maturation (Fig. 2, step 3) [51]. Malectin has been found to associate with endogenous aquaporin-2 in a large-scale proteomics study, suggestive of its involvement in glycoprotein maturation; however, the scope of proteins that act as malectin substrates is unknown [52]. Malectin lacks a canonical ER retention motif but remains ER localized at steady state [51]. Thus, it is likely that malectin is retained in the ER as a component of an unidentified complex. Presumably, malectin acts co-translationally on substrate glycoproteins, since both the glycosidase action of glucosidase I that precedes malectin binding and the subsequent trimming of glucoses by glucosidase II can occur co-translationally [5,48]. However, there is no direct evidence for the interaction of malectin with substrates as they co-translationally emerge into the ER.

The diglycosylated glycan (Glc₂Man₉GlcNAc₂) is subsequently trimmed by glucosidase II (Fig. 1 and 2, step 4). Glucosidase II is a soluble luminal enzyme that is comprised of an 110-kDa α -subunit and a 60-kDa β -subunit [53]. Glucosidase activity is localized to the α -subunit of the heterodimer, while the β -subunit in humans contains a C-terminal HDEL ER retention signal. The glucosidase II β -subunit is present in *S. cerevisiae*, yet it lacks an ER retention signal and is not required for maintaining the localization of the α -subunit [54]. In contrast, the β -subunit of *S. pombe* has a C-terminal VDEL sequence that is important for localization of the catalytic subunit [55]. Catalytic activity is exclusive to the α -subunit, since it alone displays *in vitro* enzymatic properties [56]. Glucosidase II removes two glucoses from glycans; hydrolysis of the Glc α 1,3Glc bond to generate Glc₁Man₉GlcNAc₂ glycans, and hydrolysis of the Glc α 1,3Man bond to produce a fully deglycosylated glycan (Figs. 1 and 2, steps 4 and 6). The initial cleavage of the Glc α 1,3Glc bond occurs much more rapidly than the second cleavage of the Glc α 1,3Man residues [57,58]. The Glc α 1,3Glc and Glc α 1,3Man moieties are virtually indistinguishable structurally. Rather, the differences in their catalytic cleavages are likely due to the Glc α 1,3Glc and Glc α 1,3Man cleavage epitopes residing on opposite faces of the glycan structure [48,59,60]. Since there is only a single active site within glucosidase II, the slow and nonprocessive digestion of the Glc₁Man₉GlcNAc₂ glycoform is proposed to be the result of reorientation of the active site of the α -subunit or the repositioning of the glycosylated substrate to accommodate the opposing glycosidic linkages.

Until recently the only function attributed to the glucosidase II β -subunit was in the localization of the glucosidase II heterodimer to the ER lumen via its C-terminal ER retention signal. In addition to the C-terminal ER retention signal, the glucosidase II β -subunit contains a Mannose-6 phosphate Receptor Homology (MRH) domain [48,61]. MRH domains are typically utilized for lectin-like interactions, as observed for the ER resident proteins XTP3-B and OS-9 that are involved in glycoprotein degradation [61,62]. Helenius and colleagues observed that multiple glycans present on substrates greatly accelerated the cleavage of the Glc α 1,3Glc bond. They proposed a model whereby the MRH domain of the β -subunit binds to a mannose branch of a neighboring glycan to position the α -subunit in such a way that the Glc α 1,3Glc of the substrate glycan is efficiently recognized and hydrolyzed (Fig. 2, step 4). Subsequently, the MRH

domain can recognize a mannose branch within the substrate glycan to trim the final glucose of the Glc α 1,3Man linkage (Fig. 2, step 6) [48]. A detailed biochemical study revealed that the lectin interaction of the MRH domain with substrates occurs via the C-branch mannoses [58]. This is in support of earlier work that suggested that glucosidase II catalytic activity decreased as mannoses were removed from substrate glycoproteins [63]. This model explains the observation that multiple glycans were required for the transition of substrate glycans from Glc₂ to Glc₁ glycoforms and that glucosidase II can directly compete for substrate binding with other lectins in the ER [48,58]. It is important to note that the auxiliary glycan utilized in the transition from Glc₂ to Glc₁ state does not have to be localized on the substrate glycoprotein in this model. There could be *trans* interactions occurring between glycosylated maturation machinery or other newly synthesized glycoproteins to facilitate the cleavage of the Glc α 1,3Glc bond. This model supports the findings that glucosidase II trimming occurs in a nonprocessive manner, interrupted by critical chaperone interactions.

Further studies highlight the importance of the β -subunit to glucosidase II catalytic activity. The β -subunit confers substrate specificity, is critical for the cleavage of the Glc α 1,3Glc bond and is essential for the final digestion of the Glc α 1,3Man bond to yield a fully deglycosylated substrate, although there is evidence that *S. pombe* glucosidase II α -subunit can generate fully deglycosylated substrate in the absence of the β -subunit, albeit slowly [54,55,64,65]. The trimming of glycans from Glc₂ to Glc₁ can occur in the absence of the β -subunit; however, this cleavage is greatly accelerated in the presence of the β -subunit [55,64]. The MRH domain of the β -subunit is essential for this kinetic enhancement of glucose trimming and the removal of the final glucose since functionally deficient mutations displayed inefficient and negligible conversion to the unglycosylated state. These findings lead to an amendment to the current model for the contribution of the β -subunit to glucosidase II activity. Previous work has shown that the α -subunit has a greater affinity for Glc₂Man₉GlcNAc₂ glycoforms than monoglucosylated glycans [66]. The MRH domain is critical for reorientation of the active site for the nonprocessive hydrolysis of the Glc α 1,3Glc and Glc α 1,3Man bonds [48]. In addition, the lectin-like binding of the MRH domain to the C-branch mannoses of substrate glycoproteins greatly enhances the kinetics of glucose trimming, enhancing the affinity of the alpha subunit for the conversion of substrates from a Glc₂ to a Glc₁ and eventually a fully glucose trimmed state [55,64].

The trimming of glycoproteins from Glc₂Man₉GlcNAc₂ to Man₉GlcNAc₂ does not occur in a processive manner. The exposure of this innermost glucose initiates interactions with a discrete subset of chaperones including calnexin and calreticulin that aid in the folding of secretory glycoproteins (Figs. 1 and 2, step 5) [67–70]. The lectin nature of the MRH domain of glucosidase II competes with these lectin chaperones for binding to substrates, controlling the persistency of their protective interaction [58].

3. The lectin chaperones and their associated oxidoreductase, ERp57

Calnexin and calreticulin are glycan-dependent molecular chaperones that promote proper folding in substrate proteins by stabilizing folding events, preventing aggregation, and facilitating the catalysis of disulfide bond formation [71–74]. Monoglucosylated N-linked carbohydrates (Glc₁Man_xGlcNAc₂) created by the sequential action of glucosidases I and II are recognized by these carbohydrate-binding chaperones (Fig. 2, step 5). Calnexin is a type I membrane protein and is comprised of a single globular carbohydrate binding domain and an extended proline-rich repeat domain, termed the P-domain [75]. Similarly, calreticulin contains both a carbohydrate binding and P-domain, but is a soluble protein with a C-terminal ER retention motif [76,77]. These chaperones bind monoglucosylated proteins in a Ca²⁺-dependent fashion [75,78,79]. Binding of the lectin chaperones aids in

glycoprotein folding by slowing folding events and allowing them to occur in a controlled manner [69,71]. This is also evident from studies in calnexin and calreticulin deficient cell lines, where folding was found to be accelerated; however, the subsequent folding efficiency was diminished [80].

Lectin chaperone association represents a critical constraint for glycoprotein folding. Studies using ribosome-arrested chains have found that there is a predetermined order of events when nascent glycoproteins first enter the ER lumen through the Sec61 translocon. The membrane-bound calnexin is found in close association with the translocon complex. Phosphorylation of the cytoplasmically exposed C-terminal tail of calnexin has been proposed to regulate its association with the ribosome/translocon complex [81]. Calnexin interacts initially with the nascent chain, while associations with the soluble calreticulin can closely follow depending upon the organization of glycans on the substrate [18,19]. There is also a coupling between the lectin chaperone system and BiP, the ER-resident Hsp70 family member. BiP interacts with hydrophobic stretches of newly synthesized protein prior to the addition of hydrophilic N-glycans [19,82]. The distance the glycans are located from the N-terminus can dictate whether BiP initially interacts with a nascent chain co-translationally [83]. BiP is less likely to be involved in the maturation of glycoproteins that contain glycans near the N-terminus since they are hydrophilic thus inhibitory for BiP binding and they support the early diversion to the lectin chaperone system [19,83,84].

The topology of the lectin chaperones and their substrate is also involved in determining which chaperones bind a particular substrate. Calnexin is situated to interact with membrane proximal glycans, either on membrane-associated proteins or with proteins still entering the ER lumen. Since calreticulin is a soluble protein, it is organized to interact with glycans that emerge deeper into the ER lumen, on either membrane or soluble substrates [85–87].

The lectin chaperones can also facilitate disulfide bond formation and isomerization on substrate proteins via their noncovalent association with the oxidoreductase ERp57 [73,74,88,89]. ERp57 is an ER resident protein disulfide isomerase family member with four thioredoxin domains, two of which contain catalytic Cys pairs. It docks on the lectin chaperones through an interaction of a positively charged patch on a noncatalytic thioredoxin domain and the negatively charged tip of the extended P-domain of the lectin chaperones [76]. It is thought that there is very little free ERp57 in the ER lumen since it predominantly exists in a 1:1 association with each lectin chaperone. In addition, there is a secondary pool associated with tapasin, a protein involved in class I antigen assembly and processing, however this localization appears to be independent of ERp57 redox function [90–92].

Disulfide bond formation represents an additional constraint imparted upon nascent glycoproteins during co-translational folding. Disulfides can limit the conformational flexibility, increase structural rigidity and stabilize proteins once they are exposed to the harsh extracellular environment. Disulfides can also be used as transitory elements during folding, ensuring that a critical folding intermediate is reached, and subsequently isomerized to achieve native conformations [93,94]. In conjunction with the lectin chaperones, ERp57 can perform oxidation, reduction, and isomerization reactions of Cys pairs in glycoprotein substrates [95]. ERp57 has been found mainly in a reduced form in cells at steady state [96,97]. The reduced form can only perform isomerization and reduction reactions for substrate proteins. However, it has been found associated with ERO1 α , the protein that generates oxidizing equivalents in the ER, and ERp57 has been shown to possess oxidase activity *in vivo*, suggesting that it is a fully functional oxidoreductase [95,98]. While the deletion of ERp57 is not lethal to cells, it is essential for mouse development as knockout animals die *in utero* [99,100]. There could be multiple PDI family proteins that

can substitute for ERp57 in its absence, since there are currently nearly 20 ER-resident members [101]. In recent studies of ERp57, it has been shown to be intimately involved in the late-stage rearrangement of disulfides in complex glycoproteins [100]. This highlights an additional mode of glycoprotein folding that occurs after nascent proteins have fully emerged into the ER lumen.

The folding of glycoproteins generally occurs, at least for distal interactions, after their release from the lectin chaperones that is triggered by glucosidase II-mediated cleavage of the final glucose [69,71]. Glycoproteins that have failed to achieve their native conformations following their release into the ER lumen are subjected to a quality control process [30,102]. If non-native conformations persist, the quality control sensor GT1 (UDP-glucose: glycoprotein glucosyltransferase 1) detects these instabilities and transfers a glucose back onto deglycosylated glycoproteins, regenerating monoglucosylated glycans (Fig. 2, step 8) [103–106]. These reglucosylated substrates then re-associate with the lectin chaperones and ERp57 to reinitiate folding events in a process termed the ‘calnexin cycle’ (Fig. 2, step 9) [68–70,104]. Persistent binding to the lectin chaperones assists protein folding, prevents aggregation through sequestration, retains immature or unassembled structures in the ER and potentially aids in the sorting of malformed substrates for degradation [72,107–109].

4. GT1 reglucosylation and quality control of glycoprotein maturation

GT1 is a primary folding or quality control sensor of the ER. It is currently the only known ER enzyme that alters the glycan composition of a protein based on structural features of the modified protein [104–106,110–112]. *In vitro* studies have revealed that GT1 detects malformed glycoproteins via surface-exposed hydrophobic patches, indicative of protein immaturity or misfolding [104,106]. The selectivity of GT1 for exposed hydrophobic stretches in substrate proteins allows for discrete malformed regions to be modified by reglucosylation of proximal glycans, addressing protein misfolding at the domain level (Fig. 3B, steps 3 and 4) [110,113]. Biophysical studies have demonstrated that GT1 targets nearly native molten globule proteins [111,114]. GT1 has the ability to distinguish minor structural perturbations, since *in vitro* reglucosylation increases significantly with the introduction of aberrant structures [113,115]. In addition, GT1 substrates may not necessarily be malformed at the level of monomers, since GT1 can target and reglucosylate orphan oligomeric subunits of soybean agglutinin that exhibit native conformations *in vitro* [116]. Finally, a cellular study revealed that the folding sensor capabilities of GT1 can function in the case of natively folding substrate or when protein folding is transiently or terminally disrupted (Fig. 3B, steps 6 and 7) [117].

GT1 is a 174-kDa glycoprotein comprised of two main functional domains [118,119]. The N-terminal domain of GT1 contains nearly 75% of its primary sequence and functions as the sensor of substrate misfolding, while the C-terminal domain contains the glucosyltransferase catalytic activity. The C-terminal domain is highly conserved amongst species (65%) and is homologous to proteins of the glucosyltransferase family 8 (Fig. 3A). The large N-terminal domain has a much lower similarity between species that may indicate a means of substrate selection, however domain swapping experiments of the *S. pombe* and *D. melanogaster* glucosyltransferases resulted in functional reglucosylation activity on misfolded substrates [119]. Despite numerous efforts, a structure of GT1 remains elusive.

GT1 catalyzes the transfer of glucose from the donor UDP-glucose to the terminal A branch mannose of misfolded glycoproteins, regenerating the Glc α 1,3Man signal (Fig. 1) [120]. This step occurs after the initial recognition of misfolding, as determined by *in vitro* competition assays, where saturating levels of nonglycosylated misfolded protein could prevent reglucosylation of a glycosylated

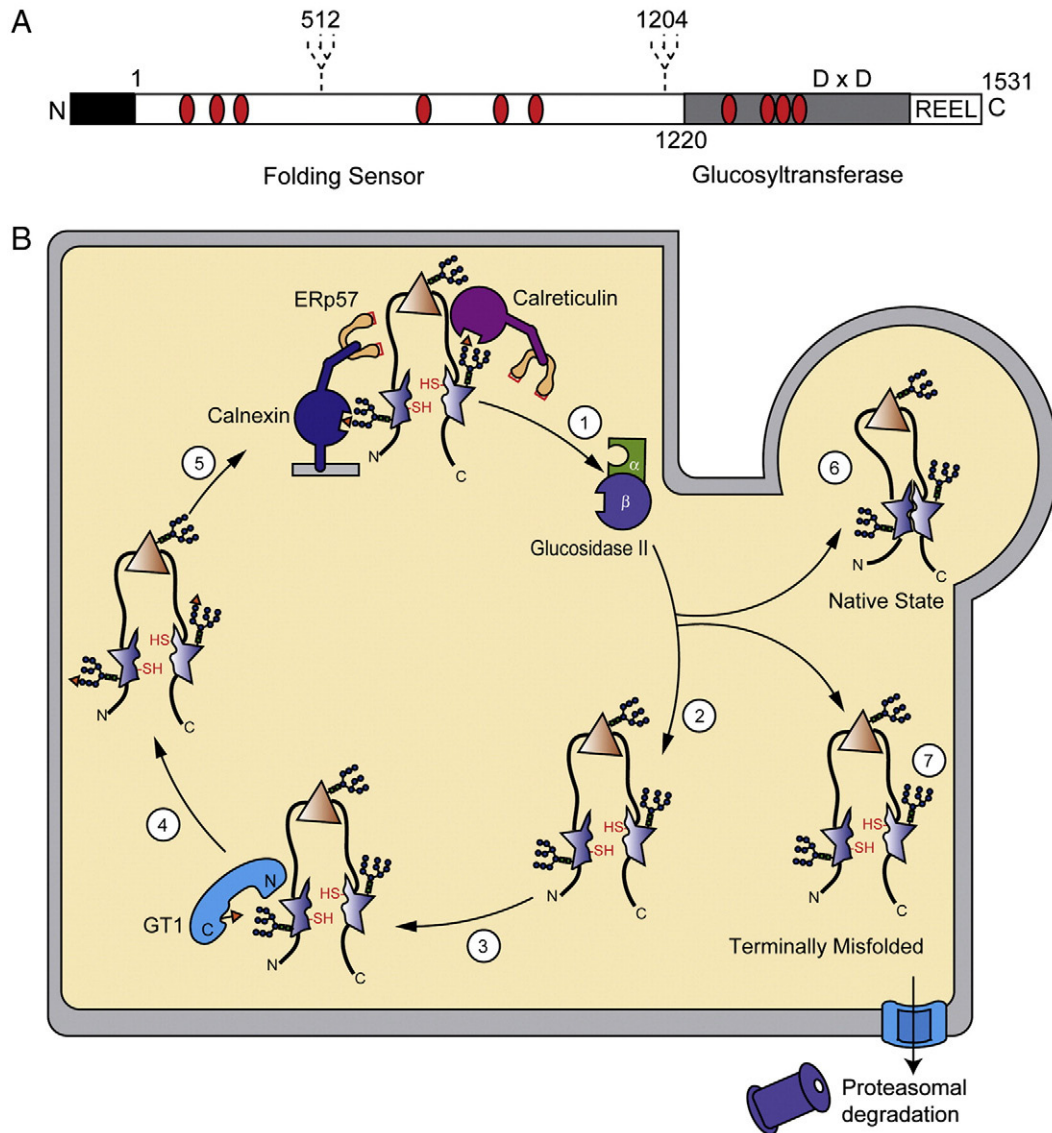


Fig. 3. Model of GT1 reglucosylation of a malformed glycoprotein. (A) Schematic representation of human GT1. Human GT1 is a 174-kDa protein with the mature protein consisting of 1531 amino acids after signal sequence cleavage. The N-terminus contains the protein recognition folding sensor domain, which represents 75% of the primary sequence and the cleavable signal sequence (black). The C-terminal domain contains the catalytic glucosyltransferase activity and the ER-retention signal R-E-E-L. In addition, the positioning of the two hypothetical glycosylation sites in human GT1 are depicted as dashed lines and Cys residues are marked with red ovals. The Asp-X-Asp motif is shown in the C-terminal domain (D x D). (B) Schematic of GT1-mediated reglucosylation in the ER. Glycoproteins are extracted from the lectin chaperone cycle by glucosidase II, which removes the last glucose in the A-branch (step 1). The chaperone released protein can attempt to fold to its native state, with certain domains being properly folded (triangle) and others being non-native or malformed (star) (step 2). GT1 can reglucosylate glycans situated near these non-native domains through docking to exposed hydrophobic patches (step 3). The substrate glycoprotein is now monoglucosylated (step 4). The newly reglucosylated protein can re-enter the calnexin and calreticulin binding cycle (step 5). The affinity of the lectin chaperones will decrease as the glycan is further trimmed by mannosidases and the protein will either be able to attain its native functional form (step 6) or be designated for degradation (step 7).

substrate [113]. Regions near the C-terminus of GT1 were found to be strictly conserved amongst species and other glucosyltransferase enzymes. Mutation of the Asp-X-Asp motif, an amino acid sequence critical for glucosyltransferase activity nearly completely inhibited reglucosylation (Fig. 3A) [121,122]. Initially, it was believed that the Asp-X-Asp motif was not involved in catalysis; rather it was utilized in binding the UDP-glucose donor. However, recent evidence from studies on other glucosyltransferases has revealed that the Asp-X-Asp motif is involved in metal coordination that is required for UDP-sugar docking and indirectly enhances catalytic activity [123]. The actual catalytic residues of GT1 are currently uncertain. In order to complete the transferase reaction, GT1 docks to the base N-acetylglucosamine and in more detailed chemical assays, has been shown to interact with the Man₁GlcNAc₂ base unit [105,124].

Both mice and humans have two distinct isoforms of the glucosyltransferase termed GT1 and GT2 [125]. Human GT1 and GT2 share 55% identity in their amino acid sequences; however, the majority of this high degree of identity is localized to the C-terminal catalytic domain (83%) [118,125]. The cellular localization of GT1 and GT2 is identical when overexpressed, but differences arise in their ability to reglucosylate misfolded glycoproteins. Functional protein chimeras created from the N- and C-terminal domains of GT1 and GT2 only resulted from the combination of the GT1 N-terminal domain and either the C-terminal catalytic region of GT1 or GT2 [118]. Both GT1 and GT2 have the ability to facilitate glucose transfer but only GT1 displays an affinity for misfolded conformers of substrate proteins. Additionally, only GT1 expression is upregulated by the unfolded protein response (UPR), supporting the importance of GT1 for protein folding in the ER [118,125].

The localization of GT1 within the ER lumen has important implications in its function. Unstable non-native conformers can have deleterious effects on the successful maturation of newly synthesized proteins in the active folding environment. Thus, it is likely that the 'calnexin cycle' takes place in an environment somewhat secluded, so that active folding events can continue uninterrupted. GT1 reglucosylation occurs post-translationally, placing its activity outside of the crowded confines of the ER translocon (Fig. 2, step 8 and Fig. 3B) [117]. GT1 has been localized to late stage folding suborganellar regions in the ER by indirect immunoelectron microscopy and quantitative proteomics [126,127]. Since not all substrates modified by GT1 are capable of achieving a native fold, it would be beneficial for degradation machinery to reside in a similar locale. Indeed, a component of the putative retrotranslocon pore, Derlin-1, is also primarily located in smooth ER membranes [127].

GT1 may not act alone in glycoprotein quality control as recently a selenocysteine-containing oxidoreductase of 15-kDa (Sep15) was found to bind GT1 with high affinity in a 1:1 complex ($K_d < 20$ nM). GT1 and Sep15 also appear to share a common subcellular localization and the ability to be transcriptionally upregulated by stress [127,128]. The binding of Sep15 to GT1 does not involve any of its catalytic oxidoreductase residues, potentially leaving these available for interaction with GT1 substrates [129,130]. A role for Sep15 in rearranging non-native disulfide bonds in an effort to reduce complications for the calnexin cycle can be envisioned. In addition, the combination of the misfolding sensor capabilities of GT1 and the reduction activity of Sep15 could be involved in preparing terminally malformed proteins for degradation.

The positioning of glycans on a substrate is important for both lectin chaperone binding and GT1-mediated reglucosylation. *In vitro* studies using a glycopeptide library found that GT1 reglucosylation occurred most frequently on glycopeptides that contained dual proximal hydrophobic patches located C-terminal to the glycan [112]. A more recent *in vitro* study used synthetic glycans and substrates to demonstrate that moving the hydrophobic moiety closer to the glycosylation site increased GT1 activity [124]. A cellular study determined that GT1 specifically targeted glycans located on the membrane proximal slow folding domain of *influenza* hemagglutinin to direct lectin chaperone post-translational rebinding to the immature or aberrant region [117]. GT1 can utilize this quality control selection process to play roles in assisting native protein folding events, triggering prolonged chaperone interactions in difficult to fold regions (Fig. 3B, step 4). Additionally, it can drive persistent chaperone association to retain misfolded proteins for additional rounds of folding or delivery to degradation machinery. This adaptability and response towards many folding situations is why GT1 is so critical to secretory protein maturation in higher order organisms and likely explains why its knockout is embryonic lethal in mice [131,132]. However, GT activity is not found in all organisms as it is missing in *S. cerevisiae* [133]. There is likely a restricted population of proteins that require an intact lectin chaperone binding cycle and some of these proteins might be needed for animal development and survival. The complexity and load of secretory cargo of multicellular organisms far exceeds that for unicellular organisms. Extracellular materials are frequently large complex multidomain glycosylated proteins with intricate disulfide networks. These types of proteins are strong candidates for utilizing and requiring an intact lectin chaperone binding cycle for efficiently acquiring their native states.

Outlook

The proper folding of secretory proteins within the confines of the ER is governed by a number of covalent and noncovalent constraints that guide the protein towards native state conformations. The trimming of glucoses from the N-glycans of glycoproteins triggers a

cascade of assistive interactions with chaperones and their associated oxidoreductase. One recently discovered lectin, malectin, provides an interesting example of temporal interactions with glycosylated substrates. Malectin interacts with Glc2 glycoforms, a glycan moiety that was thought previously to either be inert during protein maturation or provide a delay before binding to calnexin and calreticulin. It will be important to determine if malectin interacts with substrates co-translationally, and how its role in preceding calnexin and calreticulin binding assists glycoprotein folding.

The cleavage of mannoses also occurs in the ER. In functional contrast to the relation of glucose trimming to folding, loss of mannoses is thought to act as a signal for targeting for transport or degradation [29,47,134,135]. A model whereby mannose trimming signals for degradation is attractive, since mannose-trimmed glycoforms display a decreased affinity for the machinery associated with proper folding, such as glucosidase II, the lectin chaperones, and GT1 [55,64,105,136,137]. However, recent studies have revealed that the mannose trimming may also occur on the quality control machinery, providing an indirect signal for localizing an aberrant substrate that is bound to an ERAD receptor to be recruited by the membrane retrotranslocation machinery [138–140].

Recent studies have elucidated a further level of complexity in the involvement of MRH domains in glycoprotein maturation. The β subunit of glucosidase II contains an MRH domain that binds fully mannosylated glycoforms [64]. However, the quality control degradation receptors OS-9 and XTP3-B utilize MRH domains to bind mannose-trimmed species en route to proteasomal degradation [138,141–143]. How these MRH domains are functionally distinct remains unclear, since mutations of conserved residues are detrimental to the binding capacity of both classes of MRH domain-containing proteins.

The effect of glycans on protein folding is location dependent. The discrete positioning of glycans along the polypeptide chain to aid in vectorial folding is an overlooked determinant for proper folding. Specific glycans amongst an array of glycans localized on a substrate can elicit isolated lectin chaperone association or trigger the degradation process [86,144,145]. In addition, glycans can be evolutionarily selected to reside near critical Cys residues to prevent nonnative covalent association and recruit the glycoprotein-specific oxidoreductase ERp57 to the site of oxidation [18]. The situation of glycosylation consensus sites near Cys residues can also control the efficiency of glycan transfer by the OST, affecting further downstream folding events [33].

The ER is the nexus of secretory protein folding in the cell. The constraints imparted on protein folding pathways within this organelle ensure a high stringency for selection of properly formed functional folds to maintain essential cellular processes and viability. Understanding the organization of the cellular machinery in the ER responsible for eliciting these constraints using improved high-resolution microscopy techniques will provide valuable insight into how the spatial organization of the ER helps control the temporal associations with nascent chains along the maturation assembly line. Advances in our understanding of the regulation and administration of covalent and noncovalent constrictions during protein maturation in the ER will further our knowledge of these processes and provide insight into how they can be manipulated to provide potential therapies for protein misfolding diseases.

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